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Interaction of Phosducin-like Protein with G Protein βγ Subunits*

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Phosducin-like protein (PhLP), a widely expressed ethanol-responsive gene (Miles, M. F., Barhite, S., Sganga, M., and Elliott, M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* 90, 10831–10835), is a homologue of phosducin, a known major regulator of Gβγ signaling in retina and pineal gland. However, although phosducin has a well characterized role in retinal phototransduction, function of the PhLP remains unclear. In this study we examine the ability of PhLP to bind Gβγ dimer *in vitro* and *in vivo*. Using PhLP glutathione *S*-transferase fusion proteins, we show that PhLP directly binds Gβγ *in vitro*. Studies with a series of truncated PhLP fusion proteins indicate independent binding of Gβγ to both the amino- and C-terminal halves of PhLP. Protein-protein interactions between Gβγ and PhLP are inhibited by the α subunit of G_o and G_{i3}, suggesting that PhLP can bind only free Gβγ. Finally, we show that PhLP complexes, at least partially, with Gβγ *in vivo*. Following overexpression of epitope-tagged PhLP together with $G\beta_1\gamma_2$ proteins in COS-7 cells, a PhLP-Gβγ complex is co-immunoprecipitated by monoclonal antibody directed against the epitope tag. Similarly, polyclonal anti-PhLP antibody co-precipitates endogenous PhLP and Gβγ proteins from NG108-15 cell lysates. These data are consistent with the hypothesis that PhLP is a widely expressed modulator of Gβγ function. Furthermore, because alternate forms of the PhLP transcript are expressed, there may be functional implications for the existence of two Gβγ-binding domains on PhLP.

Heterotrimeric guanine nucleotide-binding proteins (G proteins) play a major role in transmembrane signaling processes by transducing extracellular signals from the superfamily of heptahelical cell surface receptors to their appropriate intracellular effectors (1, 2). In its trimeric form, Gαβγ is inactive, and the Gα subunit binds a molecule of GDP. Upon ligand binding, the receptor catalyzes the exchange of GDP for GTP on Gα that causes its activation and dissociation from the tightly bound Gβγ complex.[1] Inactivation and reassociation of the heterotrimer is initiated by the hydrolysis of bound GTP into GDP by an intrinsic GTPase activity of the Gα subunit. It is now known that both the free GTP-bound Gα and the Gβγ dimer can bind and regulate downstream effectors including adenylyl cyclases, phospholipases, and ion channels, and thereby modulate second messenger levels and ion flux (3).

The discovery of several specific Gβγ binding proteins has recently shed light on new roles for Gβγ in the propagation and termination of cellular signaling. The dimer has been shown to recruit β-adrenergic receptor kinase (βARK)[2] to its membrane-associated receptor substrate and thus initiate receptor desensitization (4, 5). This process occurs via direct binding of Gβγ to the C terminus of a putative pleckstrin homology domain on βARK (6). Furthermore, the responsiveness of G protein-regulated signaling systems may be directly modulated through the interaction of Gβγ subunits with intracellular regulatory proteins. For instance, phosducin, a phosphoprotein mainly expressed in the retina and pineal gland, inhibits the phototransduction cascade by scavenging βγ subunits of the G protein transducin (G_t), thus preventing their reassociation with the $G_t\alpha$ subunit (7, 8). Because phosducin has a higher affinity for $G_t\beta\gamma$ than does $G_t\alpha$, it has been suggested that the formation of the phosducin/$G_t\beta\gamma$ complex is a major factor regulating photoreceptor responsiveness (9). From *in vitro* binding and co-transfection assays, it was proposed that phosducin may also compete with other targets for Gβγ binding, such as βARK and phospholipase C type β2 (10, 11).

We recently isolated a rat brain cDNA encoding a phosducin-like protein (PhLP), which has 65% amino acid homology to phosducin (12). We also described several 5′-end splice variants that generate two predicted isoforms of the protein: PhLP long (PhLP) of 301 amino acids containing the entire coding sequence and PhLP short ($PhLP_S$) of 218 amino acids missing the first 83 N-terminal residues of PhLP (12, 13). Based on sequence homology with phosducin, we have suggested that PhLP proteins regulate Gβγ signaling in nonretinal tissues. In favor of this hypothesis, a recent report showed that recombinant $PhLP_S$ inhibits several Gβγ functions *in vitro* (14). Interestingly, these authors suggested that unlike phosducin (11, 15), the N terminus of PhLP was unlikely to contain a Gβγ-binding domain.

To more directly characterize the interaction of PhLP with Gβγ, we studied PhLP binding to Gβγ both *in vivo* and *in vitro*. Our results here, using *in vitro* binding studies with a series of truncated PhLP/glutathione *S*-transferase (GST) fusion proteins, show that PhLP binds βγ through a bipartite binding domain. The Gβγ-PhLP interaction was confirmed by co-immunoprecipitation of the complex from cell lysates. Our findings support the hypothesis that PhLP can modulate Gβγ function

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[1] Gβ and Gγ are thought to exist as an obligate complex and are thus referred to here as Gβγ even in instances where direct interaction may only involve Gβ.

[2] The abbreviations used are: βARK, β-adrenergic receptor kinase; DMEM, Dulbecco's modified Eagle's medium; GST, glutathione *S*-transferase; HA, hemagglutinin; PhLP, phosducin-like protein; $PhLP_S$, phosducin-like protein, short form ($PhLP^{84–301}$); PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.

(14) in many tissues through direct protein-protein interactions. Regulation of PhLP/Gβγ interactions could be an important factor in controlling G protein signaling.

EXPERIMENTAL PROCEDURES

Materials—PhLP and $PhLP_S$ cDNAs were cloned in our laboratory (12). The GST-phosducin construct was obtained from Dr. Cheryl Craft (University of Southern California). Gβγ and $G_o\alpha$ proteins purified from bovine brain were kindly provided by Dr. Eva Neer (Brighman and Woman's Hospital, Boston, MA). $G\beta_1$ and $G\gamma_2$ expression vectors were kind gifts from Dr. H. Bourne (University of California at San Francisco). Purified recombinant $G\beta_1\gamma_2$ was generously provided by Dr. Rene Onrust in the Bourne laboratory. Recombinant $G_i\alpha_3$ protein was from Calbiochem.

DNA Constructs—The full-length PhLP (amino acids 1–301) as well as different regions of the protein corresponding to amino acid residues 84–301 (referred to as $PhLP_S$), 1–167, 1–115, 1–70, 50–167, 84–167, 161–301, and 200–301 were expressed as GST fusion proteins. DNA fragments encoding PhLP and its derivatives were amplified by polymerase chain reaction using rat PhLP cDNA as template and 5′- and 3′-primers containing *Bam*HI and *Eco*RI sites, respectively. The amplified fragments were ligated in frame with the 3′-end of the coding region of GST into *Bam*HI and *Eco*RI sites of the pGEX-2T vector (Pharmacia Biotech Inc.). The resultant constructs were verified by DNA sequencing using the chain termination method (Sequenase version 2.0, U. S. Biochemical Corp.) and used to transform *Escherichia coli* strain BL21.

An epitope-tagged PhLP expression vector was generated by fusing an 8 amino acid peptide from the hemagglutinin (HA) of influenza virus to the C terminus of PhLP. Sense and antisense oligonucleotides corresponding to the HA epitope (YDVPDYAS), flanked by a 5′ *Eco*RI site and a 3′ *Not*I site, were synthesized, annealed, and inserted into pcDNA3 (Invitrogen) between *Eco*RI and *Not*I sites. The full-length PhLP coding sequence, amplified as described above, was then ligated between the *Bam*HI and *Eco*RI sites of the modified pcDNA3 vector, so as to fuse the PhLP C terminus in frame with the 5′-end of the HA tag.

Expression of GST Fusion Proteins and Gβγ Binding Assay—Fusion protein expression was induced with 0.1 mM isopropyl-1-thiol-β-D-galactopyranoside for 90 min, and the proteins were solubilized and purified on glutathione-Sepharose 4B resin (Pharmacia) by the Sarkosyl method (16). In a typical binding assay, following immobilization on glutathione-agarose beads, fusion proteins at a final concentration of 0.5–1.0 μM were incubated with 50–100 nM Gβγ purified from bovine brain in 50 μl of phosphate-buffered saline (PBS) containing 0.01% Lubrol for 2 h at 4 °C. Following six washes in 200 μl of PBS containing 0.01% Lubrol, the beads were resuspended in SDS sample buffer and boiled for 10 min. The eluted proteins were separated with 10% SDS-polyacrylamide gel electrophoresis (PAGE) and electrotransferred onto nitrocellulose membranes using standard methods. The blots were probed with a polyclonal anti-β antiserum (1:1000; DuPont NEN) and processed using the enhanced chemiluminescense detection system (Amersham Corp.). Occasionally, blots were stripped and reprobed with a polyclonal anti-GST antiserum (1:4000; Santa Cruz).

Anti-PhLP Antiserum—The entire coding region of $PhLP_S$ cDNA was amplified by polymerase chain reaction and fused in frame with the maltose binding protein coding region in the vector pMAL-c2 (New England Biolabs). The maltose binding protein-$PhLP_S$ fusion protein migrated at approximately 72 kDa on SDS-polyacrylamide gels as expected. The fusion protein was purified to apparent homogeneity by amylose resin chromatography exactly as described by the manufacturer (New England Biolabs) and was injected into rabbits by a commercial source (CalTag) for generation of a polyclonal antiserum. This antiserum was affinity-purified over a column of GST-$PhLP_S$ coupled to CnBr-activated Sepharose 4B (Pharmacia).

Cell Culture and Transient DNA Transfection—NG108-15 cells were grown as described (12) in Dulbecco's modified Eagle's medium (DMEM) containing 10% serum$^+$ (JRH Biosciences). COS-7 cells (3×10^5 cells/well) were seeded 48 h before transfection in 6-well plates in DMEM supplemented with 10% fetal bovine serum. Cells were incubated for 5 h in serum-free DMEM with DNA plasmids premixed with lipofectAmine (Life Technologies, Inc.) and were then incubated overnight at 37 °C in DMEM containing 10% fetal bovine serum. The total amount of DNA in all transfections was 2 μg/well. When required, the empty pcDNA3 vector was used to maintain a constant amount of DNA.

Immunoprecipitation—2×10^6 transfected COS-7 cells or 4×10^6 NG108-15 cells were washed twice with ice-cold PBS and lysed in Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 2 μg/ml aprotinin and leupeptin, 20 μg/ml soybean trypsin inhibitor). After 15 min of incubation on ice, insoluble material was removed by centrifugation at $10,000 \times g$ at 4 °C for 10 min, and the lysate was precleared in the presence of protein A-agarose (Santa Cruz) for 30 min. COS-7 cell lysate was then incubated with 5 μg of monoclonal antibody 12CA5 (Boehringer Mannheim) to the HA tag, whereas NG108-15 cell lysate was incubated with 5 μg of affinity-purified polyclonal anti-PhLP or an equivalent amount of preimmune serum. The incubations were conducted overnight at 4 °C before precipitation in the presence of protein A-agarose. The immunoprecipitates were washed four times with PBS containing 0.01% Lubrol, and protein complexes were eluted in SDS sample buffer and analyzed by Western blot using the 12CA5 antibody and/or a monoclonal antibody to the Gβ1 subunit (Transduction Laboratories).

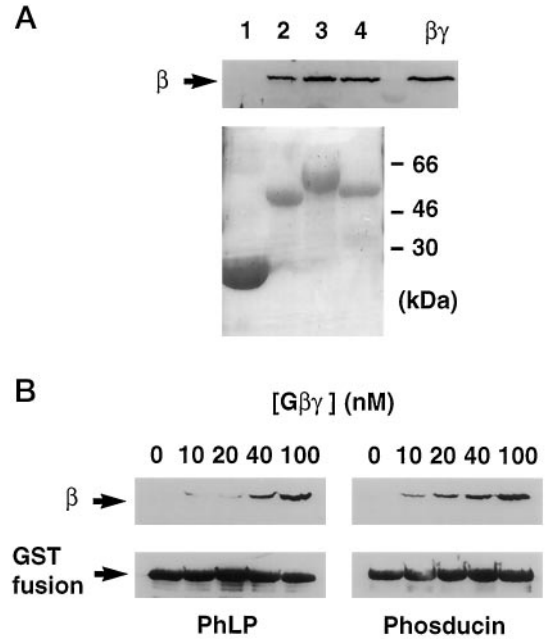

FIG. 1. **Binding of bovine brain Gβγ by GST-phosducin and GST-PhLP.** *A*, Western blot (*upper panel*) analysis of Gβγ binding to GST fusion proteins. GST fusion proteins (500 nM) or GST (1.5 μM) were incubated with 70 nM of purified bovine brain Gβγ. Bound Gβγ was fractionated by SDS-PAGE and visualized by Western blot using a polyclonal anti-β antiserum as described under "Experimental Procedures." *Lane 1*, GST; *lane 2*, GST-$PhLP_S$; *lane 3*, GST-PhLP; *lane 4*, GST-phosducin. Also shown is 500 ng of purified Gβγ alone. The *lower panel* shows Coomassie Blue staining of expressed GST fusion proteins following purification on glutathione-Sepharose resin. Approximate molecular masses (in kDa) are indicated at the *right*. *B*, titration of Gβγ binding to GST-PhLP (*left*) and GST-phosducin (*right*). GST fusion proteins were present at 1 μM in all reactions, and increasing concentrations of Gβγ were added as indicated. The amounts of GST fusion proteins and bound Gβγ were monitored by Western blot using anti-GST (*lower panels*) and anti-β (*upper panels*) antisera, respectively. The results are representative of experiments repeated at least three times.

RESULTS AND DISCUSSION

To determine whether PhLP directly interacts with Gβγ, we generated PhLP and $PhLP_S$ GST fusion proteins and examined their ability to bind Gβγ purified from bovine brain. Following immobilization of the fusion proteins on glutathione-agarose beads, Gβγ binding was detected by Western blot analysis using an anti-β antiserum. As controls, we tested Gβγ binding by the GST protein itself and GST fused to phosducin (GST-phosducin), a known Gβγ-binding protein. The fusion proteins migrate at their expected molecular weight as visualized by Coomassie Blue-staining (Fig. 1*A*, *lower panel*) and can be detected with an anti-GST antibody on Western blot analysis (Fig. 1*B*, *lower panel*). In addition, GST-PhLP proteins are recognized by an affinity-purified polyclonal antiserum directed against $PhLP_S$ (data not shown).

As previously reported by other investigators (11), GST-

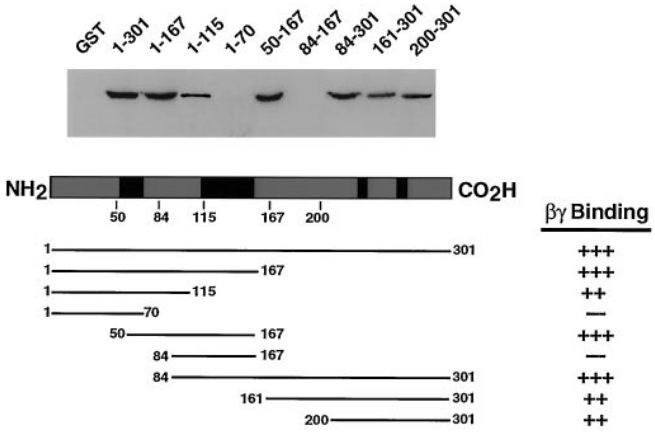

FIG. 2. **Analysis of the Gβγ binding regions of PhLP.** Western blot analysis of Gβγ binding to GST-PhLP and different deletion constructs using an anti-β antiserum (*upper panel*). GST fusion proteins (1 μM) were incubated with 50 nM $G\beta_1\gamma_2$ recombinant proteins. The *lower portion* shows schematized diagram of PhLP and deletion constructs. The predicted Gβγ interacting residues in PhLP (17) are *shaded*. The deletion constructs are illustrated together with their relative binding. The results are representative of experiments repeated at least three times.

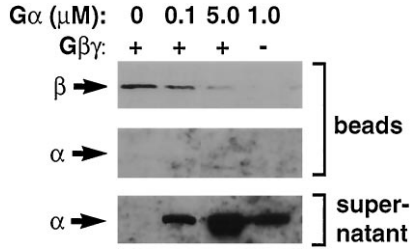

FIG. 3. **Gα inhibits binding of Gβγ to GST-PhLP.** Western blot analysis showing Gβγ binding to GST-PhLP in the presence of $G_i\alpha_3$ recombinant protein. GST-PhLP (1 μM) immobilized on glutathione-Sepharose beads was incubated in the presence of bovine brain Gβγ (35 nM) and/or in the presence of various concentrations of recombinant protein $G_i\alpha_3$, as indicated. Following 2 h of incubation, the Gβγ and the $G_i\alpha_3$ retained on the beads (*top* and *middle panels*) as well as the unbound $G_i\alpha_3$ (*bottom panel*) were detected by Western blot analysis using anti-β or anti-α antiserum (DuPont NEN). The results are representative of experiments repeated twice.

phosducin bound detectable amounts of Gβγ at a molar ratio of βγ:GST-phosducin of approximately 1:7 (Fig. 1*A*, *upper panel*). Under similar conditions, GST-$PhLP_S$ and GST-PhLP also retained Gβγ (Fig. 1*A*, *upper panel*). By contrast, GST protein did not bind Gβγ subunits even when present at a 3-fold higher concentration than the GST-PhLP proteins (Fig. 1*A*, *upper panel*), indicating that Gβγ binding by the fusion proteins is specified by the PhLP protein sequence. The affinity of phosducin for Gβγ was previously reported to be in the nanomolar range (11, 14). Under our experimental conditions, GST-PhLP appeared to have a slightly lower affinity for Gβγ than GST-phosducin but of the same order of magnitude because titration with varying amounts of Gβγ protein showed that GST-PhLP retained only slightly lower amounts of Gβγ than did GST-phosducin (Fig. 1*B*, *upper panel*).

To map the Gβγ binding domain of PhLP, we examined recombinant $G\beta_1\gamma_2$ interaction *in vitro* with GST fusion proteins containing various regions of PhLP. Fig. 2 shows an alignment of PhLP deletion constructs with the full-length PhLP. Each construct produced a protein that migrated at the expected molecular weight on SDS-PAGE (data not shown). Surprisingly, we found Gβγ binding activity of PhLP at two areas in the N- and C-terminal regions. In the N-terminal half of PhLP ($PhLP^{1-167}$), amino acids 50–115 appeared sufficient for Gβγ binding, because $PhLP^{1-115}$ and $PhLP^{50-167}$ retained Gβγ, whereas $PhLP^{1-70}$ and $PhLP^{84-167}$ did not (Fig. 2). The 50–115 region contains an 11-amino acid stretch (57–67: TGPKGVINDWR) that is perfectly conserved between PhLP and phosducin and is known to be essential for Gβγ binding by phosducin (11). Furthermore, the crystal structure of the G_t βγ-phosducin complex, reported while this manuscript was in preparation, showed that this highly conserved sequence has extensive and tight interactions with the center of the Gβ propellar (17). This conserved sequence region of PhLP (amino acids 57–67) may be important for binding of Gβγ because $PhLP^{84-167}$ totally lacked βγ binding, whereas $PhLP^{50-167}$ had essentially full binding activity. However, although the 57–67 region may be important for Gβγ binding by PhLP, additional elements seem required because $PhLP^{1-70}$ did not bind Gβγ.

Additional deletions revealed that the C-terminal half of PhLP ($PhLP^{161-301}$) also binds Gβγ. This binding activity was further localized to the C-terminal 101 residues of PhLP ($PhLP^{200-301}$) (Fig. 2). This C-terminal binding domain may explain why $PhLP_S$ ($PhLP^{84-301}$), which does not contain the 57–67 conserved sequence, still retained significant Gβγ binding activity.

The crystal structure of G_t βγ-phosducin also showed two spatially and possibly functionally distinct domains in phosducin (17). These two domains roughly correspond to the N-terminal and C-terminal halves of the molecule and do not interact with each other but both contact Gβγ. The N-terminal domain may compete with Gα, whereas the C-terminal, thioredoxin-like domain was suggested to be responsible for Gβγ translocation away from the membrane (17). Based on sequence homology, Gaudet *et al.* proposed a similar structure for PhLP and predicted the Gβγ interacting residues in this protein (17). The regions occupied by these amino acids, depicted in Fig. 2, correspond to residues 54–69 and 114–152 in the N-terminal domain and to residues 240–247 and 270–277 in the C-terminal domain. Our results are in perfect agreement with these predictions and also suggest that the N-terminal and C-terminal domains can interact with Gβγ independently. Because these domains may affect different functions of Gβγ, they might be useful tools to study different aspects of Gβγ regulation as suggested by Gaudet *et al.* (17).

Previous studies have demonstrated that the binding of Gβγ to Gα subunit inhibits its interaction with phosducin or βARK (15, 18). In contrast, the N-terminal domain of the G protein-gated K^+ channel as well as the small GTPase, ADP-ribosylation factor were shown to interact with either Gβγ alone or trimeric Gαβγ (19, 20). We found that recombinant $G_i\alpha_3$ inhibited Gβγ binding to GST-PhLP (Fig. 3). Similarly, $G_o\alpha$-GDPβS but not $G_o\alpha$-GTPγS partially abolished the interaction of Gβγ to GST-PhLP (data not shown). Together, these results suggest that only free Gβγ can interact with PhLP. Western blot analysis with a common anti-α antiserum (DuPont NEN) indicated that neither $G_i\alpha_3$ or $G_o\alpha$ was retained on GST-PhLP along with the Gβγ dimer (Fig. 3 and data not shown). In addition, $G_i\alpha_3$ by itself did not bind to GST-PhLP (Fig. 3). It should be noted that a relatively high concentration of recombinant $G_i\alpha_3$ was required to totally eliminate Gβγ binding to GST-PhLP (Fig. 3). This may reflect the fact that Gβγ interacts more tightly with PhLP than with Gα subunit, as was previously found for phosducin and $G_t\alpha$ interaction with Gβγ (17).

To demonstrate Gβγ-PhLP interaction *in vivo*, the complex was immunoprecipitated following overexpression of the proteins in COS-7 cells. For these experiments, the C terminus of PhLP was tagged with a HA epitope. COS-7 cells were transiently transfected with plasmids encoding PhLP-HA, Gβ1 and Gγ2 subunits, or a combination of these proteins. Expression of the proteins in COS-7 cells was monitored by Western blot

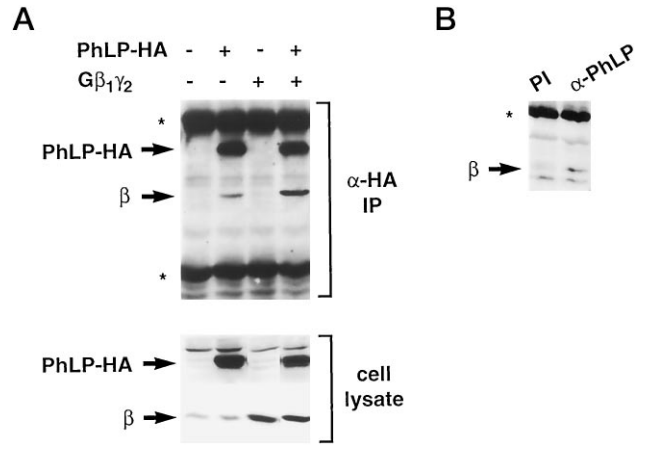

FIG. 4. **Co-immunoprecipitation of Gβγ with PhLP.** *A*, COS-7 cells were transiently transfected either alone or in combination with PhLP-HA expression vector, Gβ1 and Gγ2 expression vectors, or with empty vector as indicated. Anti-HA immunoprecipitates (*upper panel*) from COS-7 cells transfected as shown were analyzed for the presence of Gβ1 and PhLP-HA proteins by immunoblotting with anti-Gβ1 and anti-HA antibodies. Aliquots of the precleared whole cell lysates (*lower panels*) were also monitored for expression levels of PhLP-HA and Gβ1. 1/30 volume of each lysate was loaded on the gel. *B*, endogenous PhLP protein was immunoprecipitated from NG108-15 cell lysates using preimmune serum (*PI*) or a polyclonal anti-PhLP antibody. The precipitated proteins were then analyzed by Western blot for the presence of Gβ subunits using a monoclonal anti-Gβ1 antibody. The position of immunoglobulin heavy and light chains is indicated (*). Experiments were repeated at least twice with similar results.

analysis using monoclonal anti-HA and anti-β1 antibodies (Fig. 4*A*, *lower panels*). PhLP-HA was specifically precipitated by anti-HA antibody (Fig. 4*A*, *upper panel*). In addition, this antibody co-precipitated overexpressed $G\beta_1\gamma_2$ subunits, only in cells co-expressing PhLP-HA (Fig. 4*A*, *upper panel*). We also detected Gβ in immunoprecipitates from cells transfected only with PhLP-HA plasmid (Fig. 4*A*, *upper panel*), suggesting that PhLP-HA interacts with both overexpressed and endogenous Gβγ subunits.

Interaction of endogenous PhLP and Gβγ was examined in NG108-15 neuroblastoma × glioma cells because this cell line expresses high basal levels of PhLP.[3] Endogenous PhLP protein from NG108-15 cell lysates was immunoprecipitated by an affinity-purified polyclonal antiserum directed against $PhLP_S$. On Western blot analysis of NG108-15 cell lysates, this antiserum recognized a single band migrating at 46 kDa, the expected molecular mass for full-length PhLP protein (data not shown) (12). The antiserum also immunoprecipitated a 46-kDa protein from [^{35}S]methionine-labeled NG108-15 cells (data not shown). Anti-PhLP immunoprecipitates contained Gβ as detected by Western blot analysis (Fig. 4*B*). Preimmune serum did not precipitate Gβ (Fig. 4*B*). These results confirm the overexpression studies (Fig. 4*A*) and suggest that PhLP might exist, at least partially, as a complex with Gβγ subunits *in vivo*.

In conclusion, these studies have documented the direct interaction of PhLP with Gβγ through both *in vivo* and *in vitro* analyses. Our deletion analysis, together with the recent crystal structure of the phosducin-$G_t\beta\gamma$ complex, suggests that two potentially independent domains on PhLP interact with Gβγ. This complements prior studies on PhLP that suggested that regions beyond the N terminus were involved in inhibition of Gβγ function *in vitro* (14). Because the two domains of PhLP are predicted to interact with functionally different regions of Gβ (17) and we have previously shown the existence of multiple forms of the PhLP transcript, it is tempting to speculate that alternate forms of PhLP might produce distinct changes in Gβγ signaling. For example, $PhLP_S$ contains predominantly the C-terminal Gβγ-binding domain and thus might produce different kinetics or extent of changes in Gβγ function than the full-length PhLP protein. It remains to be determined which of the diverse Gβγ cellular effects are functionally modified by PhLP-Gβγ interactions.

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[3] C. Thibault and M. F. Miles, unpublished results.

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